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Human T Lymphotropic Virus Type 1 Increases T Lymphocyte Migration by Recruiting the Cytoskeleton Organizer CRMP2

Michel Varrin-Doyer,* Adeline Nicolle,* Romain Marignier,* Sylvie Cavagna,* Claire Benetollo,* Eric Wattel,‡ and Pascale Giraudon*†

Recruitment of virus-infected T lymphocytes into the CNS is an essential step in the development of virus-associated neuroinflammatory diseases, notably myelopathy induced by retrovirus human T leukemia virus-1 (HTLV-1). We have recently shown the key role of collapsin response mediator protein 2 (CRMP2), a phosphoprotein involved in cytoskeleton rearrangement, in the control of human lymphocyte migration and in brain targeting in animal models of virus-induced neuroinflammation. Using lymphocytes cloned from infected patients and chronically infected T cells, we found that HTLV-1 affects CRMP2 activity, resulting in an increased migratory potential. Elevated CRMP2 expression accompanies a higher phosphorylation level of CRMP2 and its more pronounced adhesion to tubulin and actin. CRMP2 forms, a full length and a shorter, cleaved one, are also affected. Tax transfection and extinction strategies show the involvement of this viral protein in enhanced full-length and active CRMP2, resulting in prominent migratory rate. A role for other viral proteins in CRMP2 phosphorylation is suspected. Full-length CRMP2 confers a migratory advantage possibly by preempting the negative effect of short CRMP2 we observe on T lymphocyte migration. In addition, HTLV-1–induced migration seems, in part, supported by the ability of infected cell to increase the proteosomal degradation of short CRMP2. Finally, gene expression in CD69+ cells selected from patients suggests that HTLV-1 has the capacity to influence the CRMP2/PI3K/Akt axis thus to positively control cytoskeleton organization and lymphocyte migration. Our data provide an additional clue to understanding the infiltration of HTLV-1–infected lymphocytes into various tissues and suggest that the regulation of CRMP2 activity by virus infection is a novel aspect of neuroinflammation. The Journal of Immunology, 2012, 188: 1222–1233.
During neuroinflammation, leukocytes emigrate from the bloodstream to the CNS across the blood–brain barriers through either endothelial cells of inflamed meningeal veins or epithelial cells of the choroids plexuses (16, 17). The continuous T lymphocyte extravasation, migration into, and crawling within neural tissue are orchestrated by the interplay of multiple adhesion receptors whose expression and avidity are modulated by cytokines and chemokines (18–20). Activation of G protein-coupled receptor by chemokines induces a motile phenotype characterized by the formation of lamellipodia at the front of migration and uropod at the rear of T lymphocyte (21, 22). In parallel, a drastic reconfiguration of the microtubule and intermediate filament cytoskeleton allows lymphocyte locomotion. However, the mechanism associated with lymphocyte motility per se is not totally defined. To extend our knowledge in this field, we recently focused our work on the phosphoprotein collapsin response mediator protein 2 (CRMP2), first described to modulate the microtubule reorganization during neural growth cone advance under semaphorin signal (23). Our experiments have highlighted the importance of CRMP2 in immune cells, notably in migratory activity, in both physiological and neuroinflammatory situations. We showed that CRMP2 was expressed in virtually all PBMC and had a crucial role in T lymphocyte polarization and migration (24). Investigation of the molecular mechanisms underlying CRMP2 activity in T lymphocytes indicated that CRMP2 transduced CXCL12/SDF1 chemokine signal. Differential phosphorylation by glycosgen synthase kinase-3β and Yes was supposed to modulate the contribution of CRMP2 to cytoskeletal reorganization during chemokine-induced T cell migration (25). In addition, we showed that in mouse models of virus-induced neuroinflammation, the elevated CRMP2 expression in blood lymphocytes correlated with CNS infiltration and clinical signs, suggesting the potential use of CRMP2 as a peripheral indicator of neuroinflammation (26). Extending our analysis to human, we detected high CRMP2 expression in T lymphocytes of HTLV-1–infected patients suffering from neurologic disease compared with asymptomatic virus carriers (24). We now examined whether and how HTLV-1 modifies T cell motility through alteration of CRMP2 activity. The present data demonstrate that the virus enhanced T lymphocyte migration by sustaining the presence of active CRMP2 form in infected cells, in part through Tax activity. More generally, we point to the mechanisms exploited by a virus to intensify, in infected patients, the migratory potential of T lymphocyte.

Materials and Methods

Cells

Study was performed on CD4+ T cell clones generated by platting PBMC from two HTLV-1–infected patients (0.1 cell/well, 105 CEM, Jurkat/JK, and HTLV-1–infected T cells lines C8166, C91PL, HUT-102, M92, and M94, T cell lines were cultured without IL-2. Cell selection: early-activated CD69+ cells were selected from healthy blood donor (HD) and HAM/TSP patients using MACS beads (Miltenyi Biotec). A total of 10 × 106 PBMCs were labeled with anti-CD69 mAb (20 min, 4°C), washed in medium, and incubated (20 min, 4°C) with anti-CD69 magnetic microbeads. Cells were washed, suspended in 500 μl medium, and put through magnetic columns. Selected cells (1 × 105) were used either for RNA amplification and microarray analysis, for quantitative PCR (qPCR) analysis on unamplified RNA, or for transmission assays.

Antibodies

CRMP2-A/B, generally termed CRMP2, was detected using purified polyclonal rabbit Abs directed against peptide pep4 (24) and Tax-er ter epitopes, as previously described (29) and monoclonal C4G Ab from IBL-Japan. Control Abs were directed against the full-length (62 kDa) and cleaved (58 kDa) CRMP2. Polyclonal anti–CRMP2-A Ab was described previously (29). Ab specific of phosphorylated CRMP2 forms CRMP2-pSer522 and CRMP2-pThr509/S14 was from Kinaseus (Dundee, U.K.); anti-CRMP2-pY479 was produced as described previously (25). Anti-GAPDH Ab was from Chemicon. Ab to vimentin was from Calbiochem (IF01), to tubulin from Sigma-Aldrich (TB660), and to actin was from Santa Cruz Biotechnology (SC8432). Ab to Tax was from the National Institutes of Health (158A51–42). Small interfering RNA (siRNA) anti-CRMP2 and control were used as described previously (24).

Flow cytometry

Cells were washed in PBS and incubated (15 min at room temperature) with PE-labeled anti-CD69 (BD Pharmingen) or anti-CD4 (BD Biosciences) Rabbit anti-CD3ε 5-labeled Ab (BD Biosciences), then washed and fixed with 4% paraformaldehyde in PBS (pH 7.4). 30 min at 4°C, washed again, and processed further for intracellular CRMP2 protein detection. Purified polyclonal rabbit anti-CRMP2 Ab (pep4) has been optimized for flow cytometry [signal reduction following treatment with CRMP2-pep4 or siRNA-CRMP2 (24)]. Cells were incubated with anti-CRMP2 Ab (PBS and 0.5% saponin, 30 min at room temperature), washed (PBS 0.05% saponin), incubated with FITC-labeled goat Fab(2), anti-rabbit Ab (20 min at room temperature, Roche), washed (PBS 0.05% saponin), and analyzed by flow cytometry (EPICS XL; Beckman Coulter).

Immunofluorescence

T lymphocytes were grown on glass treated with polylysine and then fixed with cold acetone for 10 min. Cells were incubated with anti-Tax and -CRMP2pep4 Abs for 1 h at 37°C, washed, and then incubated with FITC-labeled goat Fab(2), anti-rabbit Ab (20 min at room temperature, Roche), washed (PBS 0.05% saponin), and analyzed by flow cytometry (EPICS XL; Beckman Coulter).

Tax and CRMP2 expression

T lymphocytes (4 × 106) were infected with the TRIP-Tax vector plasmid (600 ng in 50 μl), 30 min at 37°C and cultured for 2 to 30 (24). CRMP2, Tax expression, and the percentage of transfected cells were evaluated by GFP fluorescence and Tax immunodetection using flow cytometry. pSG5M-Tax1, CRMP2-FLAG-wt, and the C terminally truncated AC503 plasmids have been described previously (29, 31). For the transfection, Jurkat T cells were transfected with CRMP2-FLAG-wt, CRMP2-FLAG-AC503, empty-FLAG, and empty-GFP plasmids using Amata Nucleofector technology (Köln, Germany), according to the manufacturer’s instructions. T cells were used 24 and 48 h after transfection. Transfected cells were visualized by immunostaining with anti-FLAG or anti-GFP Abs. The percentage of transfection reached 40–50%. Tax knockdown was performed using lentiviral short hairpin RNA (shRNA) –tax anti-Tax cloned in pKLO vector, as reported previously (32). Briefly, infected T cell line HUT102 was treated as follows: cell medium was changed 1 h before treatment, and cells were plated in a U-bottom 96-well plate. Cells were then treated with Polybrene (5 μg/ml; Santa Cruz Biotechnology) and 30 min later with control lentivirus (empty pKLO) and lentivirus expressing shRNA–anti Tax (10 multiplications of infect). Medium was changed at 24 h postinfection, and cells were cultured for another 2 d.

Western blotting

Cells were lysed (20 mM Tris-HCl [pH 7.4], 10% sucrose, 1 mM EDTA, and 5 mM EGTA and Complete), subjected to ultrasound fragmentation, and protein concentration was determined using Lowry assay (Bio-Rad). Proteins (20 μg) were separated under reducing conditions by SDS-PAGE and transferred to nitrocellulose membrane (Schleider and Schuell). Proteins were immunodeected with specific Ab and then revealed by the ECL method (Pierce). GAPDH detection served as control of protein deposit.

Transmigration assay

T cell transmigration was performed in triplicate in micro-Transwell (Boyden chamber) as described previously (24). T cell preparations (4 × 106) were seeded into the upper compartment of a 3.0 μm pore Transwell insert (Corning). The lower chamber was filled with human platelet-poor plasma, and incubated for 4 h at 37°C. Transfected cells were visualized by Rhodamine anti-FLAG Ab (1/1000). After 4 h, nontransmigrated cells were removed and the remaining cells were counted by fluorescence microscopy.
10^4 cells/well) were added in the upper chamber and incubated at 37°C for 1 h and 30 min. Chemokines CXCL12 (20 ng/ml), CCL5 (100 ng/ml), and CCL2 and CXCL10 (20 ng/ml) were added in the lower compartment. The migratory T cells in the lower chambers were counted under microscopy (at least 10 fields). Data are expressed as the mean number of migratory T cells.

Real-time PCR
RNA was isolated using the use of RNA PLUS (Qiagen), residual genomic DNA was removed using Dnase I (DNase-free, Ambion), and reverse transcription was performed [500 ng total treated RNA and 100 ng oligo(dT)20 primers (33)]. cDNA was amplified by real-time PCR (Light Cycler; Roche) with FastStart DNA Master SYBR Green I (Roche). PCR were separately performed with Tax, CRMP2, and β-actin primers as follows: 15 min at 95°C (20 s, 95°C, 20 s, 60°C, and 10 s, 72°C), 48 cycles. Tax primers (J02052) were 5'-ATCCGGTACCGACCTCTCA-3' and 5'-AACACTGTAAGCTGGTATCC-3'. Human β-actin primers (NM_001101) were 5'-ACTGAGAAGCTGTAAGGTGAC-3' and 5'-GTTGACTTGGAGAGGACCTGTGG-3'. CRMP2 primers (NM_001386) were 5'-TCATCACAG-AACCTCTTGGG-3' and 5'-GAAAGTTCCTCCACTCATG-3'. Specificity of amplification was achieved by a final melting step giving a single melting peak at 83.4, 77.2, and 62°C for Tax, β-actin, and CRMP2 PCR product, respectively. The level of Tax and CRMP2 mRNA was expressed as relative units normalized to β-actin.

Gene expression profile
A transcriptomic approach was performed by the genomic platform facility ProfileXpert (Lyon, France; http://www.profilexpert.fr) on CD69+ selected cells using oligonucleotide microarrays (CodeLink Uniset human 20k bioarrays; General Electric Health Care) and Assistant Pathway software, as reported previously (34). Of the 19,881 known genes and EST surveyed in HD). Again, results corroborated observations in the microarray approach. Statistical analysis on PBMC of HTLV-1–infected patients, linking the high CD69+ cells frequency with elevated CRMP2 expression (24). To go deeper into the cell locomotion machinery of activated T lymphocytes during HTLV-1 infection, we analyzed the association between CRMP2 expression (cytometry), migratory rate (Transwell system), and gene expression profile (transcriptome) in activated cells selected from HAM/TSP patients and HD (selection on CD69 Ag). CRMP2 was higher in selected cells of patients (mean fluorescence intensity = 72.2 ± 2 versus 33.3 ± 10 in HD) and migratory rate specifically elevated (22 ± 2 versus 14 ± 2 migratory cells per hour for HD; p = 0.02). Gene expression analysis (Supplemental Table I) revealed the main presence of T lymphocytes in selected cells (CD2, CD3ε, CD7, and CD8αβ mRNA) and their activation status (CD69, CD96, and CD97). Dendritic cell markers were also detected (CD1c,d, CD58, CD86, and CD207). These two cell types were susceptible to HTLV-1 infection and have been detected in HAM/TSP patients (Ref. 35 and reviewed in Ref. 36). Several genes were associated with immune cell homing (XCL2, CXCL8, CCL4L, CCL5, CCL22, CXCL10, CXCL16, CCR6, and CCR7) and transendothelial migration (CXCR4, CD18, CD44, CD49a/VLA4, CD58, and CD164). HAM/TSP patients exhibited a distinctive profile, with 22 genes uniquely expressed and 10 others with a ≥3-fold increased expression (Table I). Elevated expression of CD69 and HLAG confirmed that lymphocytes were highly activated in HAM/TSP. In addition, several induced genes were involved in molecular pathways acting downstream chemokine receptors, notably G protein and PI3K signaling (ARHGAP8, GNA13, GRASP, GRK5, GPR68, PIK3C2A, PIK3R1, RGS1, and RGS16). Other genes have been involved in intracellular trafficking and secretion (HAMP, NUP37, NUP98, RAB28, and SENP1). This approach showed that, in an HAM/TSP patient, a global activation of pathways involved in lymphocyte response to chemokine signal was associated with high CRMP2 expression.

We further focused on cultured T cell clones and evaluated the relation between cell polarization, migratory rate, and CRMP2 expression. Polarization, the initial step of lymphocyte migration, was analyzed in 12 infected and 3 uninfected T cell clones by counting the cells exhibiting a bipolar shape (uropod formation). Lymphocytes of infected T cell clones displayed uropod at higher frequency (86 ± 6%) than uninfected clones (41 ± 7%) (p < 0.01). The migratory rate of T lymphocytes was specifically enhanced for infected T cell clones (Fig. 1C, left graph), and nonlinear regression analysis of data using a logarithmic representation indicated a degree of correlation between the migratory rate and the frequency of CRMP2+ cells in each T cell clone (R² = 0.6647) (Fig. 1C, right graph). Blockade experiment using anti-CRMP2 Ab (Fig. 1D) showed the involvement of CRMP2 in the migration and polarization of infected T cell clones (Fig. 1D1, 1D2). In addition, treatment with CRMP2 siRNA reduced the number of migrating cells (Fig. 1D3). These data confirmed the role of CRMP2 in cell polarization status and migratory capacity of infected T cell clones. To conclude, HTLV-1 infection induced a profound alteration of the T cell motility machinery in parallel of modulation of CRMP2 expression.
The viral protein Tax is involved in the modulation of CRMP2 level and lymphocyte migratory rate

We suspected the virus protein Tax to regulate CRMP2 expression in infected T cell clones, because this protein is known to maintain chronic activation of T lymphocytes and also modulates expression of viral and cellular genes (11, 37). A set of experiments solidly supported this hypothesis. Analysis of Tax at mRNA level was performed by qPCR in virus-infected T cell clones (11 analyzed) (Fig. 2). Linear regression analysis, which combined tax expression level and CRMP2+ cells frequency in each clone, revealed a degree of correlation between CRMP2 (positive cells in percentages) and tax expression level ($R^2 = 0.626$). Using RT-PCR and Western blotting, we also compared CRMP2 expression in chronically infected Tax-expressing T cell lines (C91PL, C8166/45, and HUT102 cell lines) and uninfected T cell lines (Jurkat and CEM) (Fig. 2B). Increased CRMP2 mRNA and protein levels in chronically infected T cells substantiated the association of Tax with elevated CRMP2 expression. However, Tax in T cell clones also reflected HTLV-1 proviral expression, suggesting that virus proteins other than Tax could modulate CRMP2 in infected lymphocytes. The role of Tax in modulation of CRMP2 expression level was confirmed in primary T lymphocytes transduced with HIV-based lentiviral vector (TRIP-Tax) to express Tax (Fig. 2C). TRIP-gfp was used as control (data not shown). Flow cytometry analysis detected large amount of Tax in transduced lymphocytes on day 2 (95% Tax+ cells) and elevated CRMP2+ cell frequency in parallel (77–90%). Likewise, Tax transfection in the T cell line Jurkat enhanced CRMP2 expression level, as shown by Western blotting performed at 24 and 48 h posttransfection (Fig. 2D). Thus, Tax protein per se was able to augment CRMP2 expression in T lymphocyte. Finally, to evidence a direct effect of Tax protein on T cell motility, TRIP-Tax–transduced T lymphocytes were examined for migratory property (Fig. 2E). Tax+ lymphocytes exhibited a higher migratory rate compared with lymphocyte control ($p = 0.01$). Altogether, these data implicated Tax in the motile phenotype displayed by HTLV-1–infected T lymphocytes via a modulation of CRMP2 expression level.

Virus infection affects CRMP2 phosphorylation and adhesion to cytoskeleton elements in T lymphocytes

In neural and immune cells, CRMP2 is known to mediate intracellular signaling of several membrane receptors, an activity regulated by the concerted action of diverse kinases, acting notably at the C terminus of the protein (reviewed in Refs. 25 and 38). In addition, CRMP2 activity also results from opposing activity of its two molecular subtypes, CRMP2-A and -2A/B, which occur from the alternative usage of coding exons. They indeed exert a contrastive effect on microtubule pattern and neuron morphology because CRMP2A supports axonal elongation by antagonizing to CRMP2A/B (39). We therefore investigated the consequence of virus infection on CRMP2 activity by analyzing CRMP2 subtypes

*FIGURE 1. HTLV-1 infection conferred higher CRMP2 expression and migratory rate in virus-infected T lymphocytes. Analysis by flow cytometry of T cells cloned from HTLV-1–infected patients: A, elevated frequency of CRMP2+ cells in virus-infected T cell clones; B, elevated frequency of CRMP2+ and CD69+ activated cells in total lymphocytes in infected T cell clone compared with uninfected clone (left panel); elevated frequency of CRMP2+ in CD69+ cells in infected T cell clone (right panel). C, High migratory rate for infected versus uninfected T cell clones, evaluated in transmigration assay (Transwell chambers, triplicate, 1.5-h migration) toward chemokine mixture (C, left panel). Nonlinear regression analysis (C, right panel, logarithmic representation) showed a correlation between migratory rate and CRMP2+ cells frequency in each clone. D, Dose-dependent reduction of migration (D1) and polarization (D2) (infected T cell clone tested) by treatment with anti-CRMP2 blocking Ab compared with cells without treatment (control). Reduction of cell migration for two infected T cell clones treated with siRNA anti-CRMP2 or control.*
and phosphorylated forms in chronically infected T cell lines. Western blotting was performed using polyclonal Abs directed against CRMP2-A, CRMP2-A/B (generally termed CRMP2), CRMP2-pSer522, CRMP2-pThr509, and CRMP2-pTyr479 (Fig. 3A). As expected, CRMP2-2(A/B) expression was elevated in virus-infected T cell lines. The most striking difference appeared in CRMP2-A expression, only detected in the uninfected cell line CEM. Interestingly, CRMP2-A is known for its blocking effect toward CRMP2(-A/B) in the control of neural cell polarity (39). In addition, CRMP2-pSer522, CRMP2-pThr509, and at a lesser extent, CRMP2-pTyr479, forms were enhanced in virus-infected cells, notably in productive infected cell lines, as shown by the ratio of phosphorylated forms to total CRMP2 (CRMP2-A/B) signal. Because Ser522, Thr509, and Tyr479 are target for Cdk5, GSK-3\( \beta \), and the Src kinase Yes, respectively (25, 40), we analyzed the active form of these kinases using Western blotting (data not shown). None or few modifications were observed in infected versus uninfected T cell lines. The effect of Tax protein on CRMP2 phosphorylation was also investigated using tax transfection of Jurkat cells. Tax affected the level of CRMP2 as expected (1.6-fold increase), but only weakly changed CRMP2 phosphorylation (weak increase in pSer522 form) (Fig. 3B). To confirm this aspect, the infected T cell line HUT102 was transduced with lentivirus-expressing shRNA anti-Tax and vector control, as described previously (32), and cells were examined in Western blotting with anti-pCRMP2 Abs. Tax knockdown did not lead to significant modification of phosphorylation of CRMP2 on Ser522 and Tyr479, as shown by the ratio pCRMP2/CRMP2 (0.37 and 0.35, respectively). Overall, these data showed the ability of HTLV-1 infection to change the phosphorylation status of CRMP2, thus to modify its activity. However, Tax was not involved in this posttranslational modification.

In neural cells, phosphorylation modulates CRMP2 interaction with cytoskeleton elements in response to external signals, resulting in cytoskeleton reorganization, axonal elongation, and cell migration (41, 42). We then compared CRMP2-2(A/B) expression, only detected in the uninfected cell line CEM. Interestingly, CRMP2-A is known for its blocking effect toward CRMP2(-A/B) in the control of neural cell polarity (39). In addition, CRMP2-pSer522, CRMP2-pThr509, and at a lesser extent, CRMP2-pTyr479, forms were enhanced in virus-infected cells, notably in productive infected cell lines, as shown by the ratio of phosphorylated forms to total CRMP2 (CRMP2-A/B) signal. Because Ser522, Thr509, and Tyr479 are target for Cdk5, GSK-3\( \beta \), and the Src kinase Yes, respectively (25, 40), we analyzed the active form of these kinases using Western blotting (data not shown). None or few modifications were observed in infected versus uninfected T cell lines. The effect of Tax protein on CRMP2 phosphorylation was also investigated using tax transfection of Jurkat cells. Tax affected the level of CRMP2 as expected (1.6-fold increase), but only weakly changed CRMP2 phosphorylation (weak increase in pSer522 form) (Fig. 3B). To confirm this aspect, the infected T cell line HUT102 was transduced with lentivirus-expressing shRNA anti-Tax and vector control, as described previously (32), and cells were examined in Western blotting with anti-pCRMP2 Abs. Tax knockdown did not lead to significant modification of phosphorylation of CRMP2 on Ser522 and Tyr479, as shown by the ratio pCRMP2/CRMP2 (0.37 and 0.35, respectively). Overall, these data showed the ability of HTLV-1 infection to change the phosphorylation status of CRMP2, thus to modify its activity. However, Tax was not involved in this posttranslational modification.

In neural cells, phosphorylation modulates CRMP2 interaction with cytoskeleton elements in response to external signals, resulting in cytoskeleton reorganization, axonal elongation, and cell migration (41, 42). We then compared CRMP2 subcellular localization in uninfected Jurkat and HTLV-1–infected C8166 T cell lines. Western blotting performed on membrane/endosome, nucleus, and cytoskeleton cell fractions showed, in C8166 cells, a higher recruitment of CRMP2 to the cytoskeleton compartment that paralleled its decrease in the membrane/endosomal fraction (Fig. 3C). Preferential CRMP2 adhesion to cytoskeleton in C8166 versus Jurkat T cells was also suggested by immunofluorescence analysis (Fig. 3D). Because CRMP2 is able to bind actin, tubulin, and the intermediate filament vimentin (23), we investigated whether virus infection modified CRMP2 binding to these proteins. Immunoprecipitation of tubulin, actin, and vimentin was performed on Jurkat and C8166 cells, and then, CRMP2 was evaluated in immunoprecipitates. It must be noted that CRMP2 is often expressed as a 62-kDa full-length and a 58-kDa short-processed form resulting from calpain-mediated cleavage (a 50-kDa product may appear). With Ab that recognizes the long and short CRMP2 forms, we observed a 2-fold increase in the 62-kDa full-length and a 1.5-fold increase in the 58-kDa form in C8166 versus Jurkat T cells, suggesting a higher expression of CRMP2 in infected cells. As expected, no modification of CRMP2 binding to tubulin and actin was observed in infected versus uninfected Jurkat cells. With vimentin, a slight decrease in CRMP2 binding was observed in infected versus uninfected Jurkat cells. In C8166 cells, CRMP2 binding to actin and tubulin was increased compared with Jurkat cells. In addition, CRMP2 binding to vimentin was enhanced in infected versus uninfected C8166 cells. These data suggest that HTLV-1 infection leads to a modification of CRMP2 binding to cytoskeleton elements, possibly through an increase in its expression.
short forms (CRMP2-pep4 Ab), Western blotting detected CRMP2 coimmunoprecipitation with tubulin, actin, and vimentin, as described previously. However CRMP2 pattern differed in C8166 versus Jurkat cells (Fig. 3E). Notably, the full-length CRMP2 bound the three cytoskeleton components in C8166 cells but only vimentin in JK cells. To conclude, HTLV-1 modulated the phosphorylation status of CRMP2 and its recruitment to cytoskeleton elements in T lymphocyte.

HTLV-1 acts on CRMP2 proteolytic processing, favoring the presence of active CRMP2 form in infected T lymphocytes

We have recently reported the importance of proteolytic processing of CRMP2 in controlling the protein activity in neural cells (27). The short-processed CRMP2 form (58 kDa) antagonized the full-length (62 kDa), acting as a physiological dominant-negative signal to reduce neural cell process extension. This observation and the preferential adhesion of the full-length/active CRMP2 form to actin and tubulin in infected T cells prompted us to test the hypothesis that HTLV-1 enhanced CRMP2 activity in T lymphocyte by increasing the level of the CRMP2 active form. We first tested whether short CRMP2 acted as a dominant-negative signal in immune cells by testing the migratory rate of Jurkat cells transfected with plasmid coding either for the full-length CRMP2 (CRMP2wt) or the short dominant-negative form, CRMP2Δ503, as previously reported for neural cells study (29). Cells transfected with gfp plasmid were used as control. As shown in Fig. 4A, transfection with CRMP2wt and CRMP2Δ503 had opposite effect on T cell migratory rate (enhancement versus decrease, respectively), validating in immune cells the dominant-negative effect of short CRMP2. We further examined the presence of full-length and short CRMP2 in uninfected and HTLV-1–infected T cell lines using Western blotting and CRMP2-pep4 Ab (Fig. 4B). The short (58 kDa) and full-length (62 kDa) products were detected in all T cell lines, but the full-length CRMP2 was overexpressed in infected T cells, as shown by the 58:62-kDa ratio. The role of HTLV-1 in the modification of CRMP2 processing was then evaluated in tax-transfected Jurkat cells (Fig. 4C). Western blotting showed that CRMP2 level expression was enhanced in Tax expressing cells, as expected. In addition, the 58:62-kDa ratio was strongly reduced (51 to 9, in gfp- versus tax-transfected cells), indicating that Tax promoted the full-length active form in transfected lymphocytes. To confirm this aspect, infected T cell line HUT102 was transduced with lentivirus-expressing shRNA anti–Tax, and cells were examined for CRMP2 cleavage using Western blotting. Knockdown expression of Tax protein was associated with the reduction of full-length CRMP2, as shown by the augmentation of the 58:62-kDa ratio (3:21) in control lentivirus versus shRNA anti–Tax-treated cells.

As Tax is known to regulate the function of the proteasome, notably by binding specifically to two subunits of the 20S proteasome, HsN3 and HC9 (43), the evolving 58:62-kDa ratio of CRMP2 that we observed led us to analyze CRMP2 proteasomal
degradation under Tax influence. Jurkat and C8166 cells were treated with the proteasome inhibitor PS-341 (10 nM for 24 h), allowing us to evaluate the fraction of CRMP2 protein subjects to degradation. The expression of the short and full-length forms of CRMP2 was analyzed by Western blot. We observed that the fold increase of short CRMP2 expression after PS-341 treatment was dramatically enhanced (108-fold more) in the infected cell line C8166 compared with the uninfected Jurkat one (Fig. 4D).

By contrast, the expression of full-length CRMP2 was similarly affected in the two cell lines. These results suggest that HTLV-1 infection may contribute to the modification of the 58:62-kDa ratio by enhancing the degradation of the short form of CRMP2.

To substantiate a possible link between Tax, CRMP2 proteolytic processing and T lymphocyte migration, we examined the migratory rate of Tax-transfected Jurkat cells following cotransfection with full-length or short CRMP2 (CRMP2wt and CRMP2ΔS503). Tax enhanced the migratory rate of cotransfected Jurkat cells, as expected (Fig. 4D). In addition, in the presence of Tax, the cells transfected with CRMP2ΔS503 showed no reduction of their migration ability compared with the control, contrary to what was observed with CRMP2ΔS503 alone. Interestingly, Western blotting analysis of CRMP2 showed that transfection with CRMP2ΔS503 was associated with a decreased detection of the endogenous full-length CRMP2 in Jurkat cells, whereas cotransfection with Tax restored this expression (Fig. 5B). All these data strongly suggested that the elevated migratory rate displayed by HTLV-1–infected T cell clones reported above was associated with high level of full-length/active CRMP2. This hypothesis was examined in eight infected T cell clones established from patients using CRMP-2-Cter and -pep4 Abs in Western blot analysis. Interestingly, the full-length CRMP2 was detected only in infected T cell clones (Fig. 5C). We then examined the possible association between the full-length form in T lymphocytes and their migratory rate (three infected and three uninfected T cell clones studied) following evaluation of the full-length/short (62:58 kDa) CRMP2 ratio (Fig. 5D, migration in lower panel, CRMP2 ratio in upper panel). We observed that the more favorable to the full-length form the ratio, the more elevated the migratory rate of infected T-cell clones was. Collectively, these data demonstrated that HTLV-1 infection, possibly through Tax activity, profoundly altered posttranslational processing of CRMP2, resulting in more active molecular form in T lymphocyte and elevated migration as a consequence.

**FIGURE 3.** Modification of CRMP2 phosphorylation and subcellular localization of CRMP2 in HTLV-1–infected T cell lines. A, Analyze of CRMP2 subtypes, CRMP2-A and CRMP2-(A/B), and the phosphorylated CRMP2 forms: CRMP2-pSer522, -pThr509/514, and -pTyr479, using Western blotting: loss of CRMP2-A and increase in phosphorylated forms in HTLV-1–infected cell lines compared with uninfected CEM cells (ratio to CRMP2-[A/B] signal). B, CRMP2 phosphorylated forms in tax-transfected Jurkat cells: increased CRMP2 level in transfected cells (ratio to signal in gfp cells) and CRMP2 phosphorylated on pSer522 (ratio to CRMP2 signal in gfp cells). C, CRMP2 subcellular localization in Jurkat and C8166 T cell lines: enhanced recruitment to cytoskeleton compartment in HTLV-1–infected cells C8166 cells (vimentin as control of subcellular fraction). D, CRMP2 (red color) colocalizes (orange color, arrows) with cytoskeleton elements vimentin and actin (green color) in HTLV-1–infected cells C8166. Original magnification ×500. E, Coimmunoprecipitation of CRMP2 with cytoskeleton elements tubulin, actin, and vimentin in Jurkat and C8166 T cells: modification of adhesion pattern to actin and tubulin in HTLV-1–infected cells (full-length, 62-kDa CRMP2-bound tubulin and actin only in infected cells; black arrow).
Tax and CRMP2 colocalized at uropod and cell–cell contact in HTLV-1–infected T cells

Given 1) the crucial role of CRMP2 in remodeling neural cell and T lymphocyte cytoskeleton by direct binding to microtubules (25, 44) and also in participation, as a cargo receptor, in the transport of specific vesicles (45, 46), 2) the ability of Tax protein to modulate CRMP2 activity in infected lymphocyte, in particular cytoskeleton binding (shown in this paper), 3) the capacity of infected cell to transfer Tax through a well organized cell–cell contact (the so-called virological synapse (47), and 4) the role of microtubule in T cell signaling (48), we suspected CRMP2 to bind Tax protein at specific sites in lymphocyte cytoplasm to cooperate. Preliminary analyses using immunoprecipitation and CRMP2-GST pull-down strategy were unable to detect a direct association between these proteins. Nevertheless, we further analyzed the localization of these two proteins in infected lymphocyte. Immunofluorescence study showed the presence of Tax in lymphocyte cytoplasm, as small dots and its colocalization with CRMP2 at uropod in infected T cell clone and at the site of cell–cell contact in infected/productive HUT102 T cells (Fig. 6). This suggested a possible cooperation between these two proteins for uropod formation and subsequent lymphocyte migration and for molecule/signal delivery through membrane connection.
infected T cell clones established from infected individuals, activates the lymphocyte locomotion machinery. Using virus-60–62). In this study, we describe a new strategy for HTLV-1 and phocyte entry into the CNS through the blood–brain interfaces (56, molecule adhesion, as such was suspected to facilitate T lym-

tifications. Transfection experiments directly assign a role for Tax by altering its protein cellular level and posttranslational mod-

T lymphocytes express and can release the viral protein Tax, a as a potent regulator of cellular gene transcription (reviewed in Ref. 53). Infiltrated T lymphocytes have a crucial role in pathogenesis as they constitute the major reservoir of HTLV-1 in the CNS of patients, and they can produce inflammatory cytokines and metalloproteases described for their del-terious effect on neural cells (54–56). In addition, infiltrated T lymphocytes express and can release the viral protein Tax, a strong inducer of proinflammatory cytokine secretion from the resident CNS cells (57–59).

The strategy developed by HTLV-1 to enhance T lymphocyte motility involves several aspects. HTLV-1 infection has been re-
pported to augment expression of metalloproteases and membrane molecule adhesion, as such was suspected to facilitate T lymphocyte entry into the CNS through the blood–brain interfaces (56, 60–62). In this study, we describe a new strategy for HTLV-1 and report a functional interplay between the virus and CRMP2, which activates the lymphocyte locomotion machinery. Using virus-infected T cell clones established from infected individuals, chronically infected T cell lines, and tax-transfected lymphocytes, we showed a direct role of HTLV-1 in modifying CRMP2 activity by altering its protein cellular level and posttranslational modifications. Transfection experiments directly assign a role for Tax protein in the elevated expression of CRMP2 in T lymphocytes. As a potent regulator of cellular gene transcription (reviewed in Ref. 37), Tax has the ability to control ccrmp2 expression. In fact, several transcriptional sites located on the ccrmp2 gene promoter were recently described and include a binding site for the transcrip-
tion factor Sp1 (63). Tax by itself can activate Sp1 through

direct interaction. In addition, it is known that Sp1 activity depends, in part, on repression of PP2A phosphatase, a negative regulation that may be achieved by HTLV-1 (64–67). In contrast, Tax could modulate CRMP2 expression by interfering with TGF-β super-family members, including bone morphogenetic protein (BMP). SMAD elements are present in ccrmp2 promoter and BMP2 and BMP4 can suppress the expression of CRMP2 in neural cells through direct interaction of SMAD1 and SMAD4 (68). The ability of Tax protein to block TGF-β signaling recently detected in HTLV-1–infected T cells (69) suggests a possible enhancement of CRMP2 expression by blockade of this negative regulation. It is now known that CRMP2 expression level is not sufficient to trigger cell motility. Posttranslational modifications, including phosphorylation and proteolytic processing are crucial for CRMP2 activity. We and others (40, 42, 70, 71) have previously shown that sequential phosphorylation of CRMP2 is required for semaphorin and chemokine signaling in neural and immune cells, respectively. In T lymphocyte, CXCL12/SDF1 signal decreases phosphorylation on Thr509, allowing phosphorylation on Tyr479 presumably by the kinase Yes, and induces T cell migration (25). In the current study, we showed that HTLV-1 enhanced the phosphorylation of CRMP2 on several residues, a feature associated with CRMP2 recruitment on the cytoskeleton of infected lymphocyte. In addition, analysis of gene expression profile in activated lymphocytes of HAM/TSP patients revealed the activation of T cell locomotion machinery. Altogether, this suggests that HTLV-1 infection chronically activates the molecular pathways involved in lymphocyte motility, notably those linked to CRMP2. Several observations support this hypothesis. First, CRMP2 belongs to the PI3K/Akt/GSK-3 pathway, shown essential for axon elongation in neurons (41) and for cell migration in general (72). The ability of HTLV-1 to activate PI3K/Akt signaling pathway in T lymphocyte has been demonstrated (73) and could explain the specific gene profile found in cells selected from HAM/TSP patients that involves the molecular G protein/PI3K pathway. Interestingly, CRMP2 is a binding partner of several proteins of this pathway, including Fyn, PI3KR1/p85, and Vav-1 (25, 42). Finally, Tax may bind PI3KR1/p85 (37) and influence Vav phosphorylation (74). To conclude, HTLV-1 in-
fection has the ability to influence the CRMP2/PI3K/Akt/GSK-3 axis in T lymphocyte thus to positively control the migratory activity of infected cells. The present observation on ex vivo lymphocytes complements two previous reports showing that HTLV-1 regulates G protein signaling, in particular, activates the SDF1/CXCR4 axis in T lymphocyte of ATL patients, lymphoblastoid cells from Tax transgenic mice, and HTLV-1–infected T cell lines (75, 76).

CRMP2 proteolytic cleavage is an additional posttranslational modification essential for cell motility/outgrowth. In fact, CRMP2 is expressed in neural cells as a full-length (62 kDa) and a short-processed form (58 kDa), resulting from cleavage by the calcium-dependent protease calpain (77). CRMP2 cleavage is enhanced in injured neural tissues displaying limited postinjury remodeling and neurite regeneration (78). Our demonstration that the short CRMP2 molecular form negatively regulated axon elongation could explain in part this feature (29). In the context of immune cells, we have recently identified the full-length and short CRMP2 forms in T lymphocytes (25) and showed, in the present work, that the negative regulation of short CRMP2 (CRMP2Δ503) occurs in lymphocyte migration. Interestingly, cotransfection experiments pointed out the ability of Tax protein to overcome the negative effect of short CRMP2 on T cell migration. Such a feature could have an explanation at protein level as transfection with CRMP2Δ503 dramatically reduced the full-length CRMP2 in Jurkat cells, whereas cotransfection with Tax restored this expression. Given these data, privileging the full-length/active CRMP2 form in T lymphocyte
could be a means for HTLV-1 to promote cell migration. The following observations support this hypothesis: 1) the ratio of CRMP2 forms was always more in favor of the full-length protein in infected T cells clones, tax-transfected T cells, and infected T cell lines; 2) knocking down Tax expression through lentiviral shRNA strategy resulted in decreased level of the full-length protein; 3) the full-length CRMP2 form, known as a direct modulator of microtubules (44), was bound to the microtubule protein tubulin in HTLV-1–infected T cell line and not in control T cells; and 4) the presence of full-length CRMP2 in infected T cell clones correlated with elevated migratory rate. The high level of full-length CRMP2 in infected T lymphocytes could result either of a less effective protein cleavage or a modified stability of one or another form of CRMP2. We observed that HTLV-1 infection could result in an important increase of the proteasomal degradation of the short CRMP2, supporting the modifications of the 58:62-kDa ratio observed. Although the precise mechanism of CRMP2 modulation remains unclear, it obviously appears that HTLV-1 exploits a fundamental cell regulatory mechanism to enhance lymphocyte motility.

The viral protein Tax appeared to be involved in the modulation of CRMP2 level and cleavage but not in the enhanced phosphorylation displayed by HTLV-1–infected lymphocytes, suggesting that other viral proteins can modify CRMP2 expression and activity. Nevertheless, colocalization of Tax and CRMP2 at cell sites important for cell migration (uropod) and molecule/signal delivery (membrane cell–cell contact) strongly suggests the cooperation of these two molecules to support functions that are important for HTLV-1 virus/protein transmission (79, 80). This interesting aspect of CRMP2 function in virus-infected lymphocytes remains to be analyzed.

Other neuroinflammatory/neurodegenerative diseases share the importance of infiltrating T lymphocytes in pathogenesis. A link between diffuse axonal loss, a key feature of multiple sclerosis, and the presence of meningeal T lymphocytes in spinal cord of patients was reported recently (81). In addition, recent studies assigned an unexpected role to infiltrated T lymphocytes in the pathophysiology of Parkinson’s disease and Amyotrophic lateral sclerosis (82, 83). Chronic activation of cell signaling programs that directs immune cell motility and includes CRMP2 could be a common mechanism leading to CNS infiltration and tissue damage in various neuroinflammatory diseases. This point merits to be investigated.

To conclude, our findings provide an additional clue to understanding the migration and infiltration of HTLV-1–infected lymphocytes into various tissues and suggest that the regulation of CRMP2 activity by retrovirus infection is a novel aspect of virus-induced neuroinflammation.

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